Memo To: James Hutchings, Ph.D., Toxicology Program Manager
From: Rebecca Wagner, Ph.D., Research Section Supervisor
CC: Alka Lohmann, Technical Services Director
Date December 14, 2020
RE: Method Development Summary
Method Development for Barbiturate Quantitation and Confirmation by Liquid-Liquid Extraction Using LCMSMS

Method Development Summary-Barbiturate Quantitation and Confirmation by Liquid-Liquid Extraction Using LCMSMS

The following compounds were evaluated during method development:

Target	Internal Standard
Butabarbital	Butalbital-D_5
Butalbital	Butalbital-D_5
Pentobarbital	Pentobarbital-D_5
Phenobarbital	Phenobarbital-D_5
Secobarbital	Secobarbital-D_5
Amobarbital	Amobarbital- D_5
Thiopental	
Glutethimide	

Instrumental Method Development

Initial method development was aimed to develop a quantitative method with a single dynamic range for the analysis of barbiturates in biological matrices. All target compounds were optimized on an Agilent Technologies 6430, 6460, and 6470 LCMSMS using Agilent Technologies Optimizer software. All compounds were optimized with a negative ionization polarity. Thiopental and glutethimide were unable to be optimized using the Optimizer software. Manual optimization was attempted and was unsuccessful. Therefore, thiopental and glutethimide were removed from the method. Initial method development aimed to utilize instrumental parameters from the Anti-Epileptic Drugs Quantitation and Confirmation by LCMSMS in the Toxicology Procedures Manual (Qualtrax Revision 17), with the exception of ionization polarity. The data acquisition method was a DynamicMRM method with 11 minute gradient and two minute post time. Upon gradient optimization, it was determined that amobarbital and pentobarbital shared the same precursor ions, product ions, and retention times. Optimization to achieve separation was performed.

The optimal column configuration was determined to be an Agilent Technologies Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm particle size column. The gradient was optimized to improve peak shape and achieve separation between isomers. Separation between amobarbital and pentobarbital was not achievable. Therefore, the gradient was aimed to ensure co-elution of amobarbital and pentobarbital. The mobile phase consisted of 5 mM ammonium acetate in water (Mobile Phase A) and methanol (Mobile Phase B). The optimized instrumental parameters are delineated in Table 1.

Table 1 Optimized instrumental parameters

Parameter	Setting		
Column	Agilent Technologies Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm		
Injection Volume	10 uL		
Needle Wash	20 seconds		
Flow Rate	0.7 mL/min		
Mobile Phase A	5 mM Ammonium Acetate Water		
Mobile Phase B	Methanol		
Gradient	Time (min)	% A	% B
	0.0	90	10
	9.5	55	45
	10.5	10	90
	11.5	10	90
	12.0	90	10
Post Time	1.0 minutes		
Column Temperature	60°C		

As mentioned, the instrument was utilized in negative ionization mode with DynamicMRM analysis. The mass spectrometer parameters and listed in Table 2. Additionally, Table 3 lists the precursor ions, product ions, approximate retention times, and instrumental voltages for each compound.

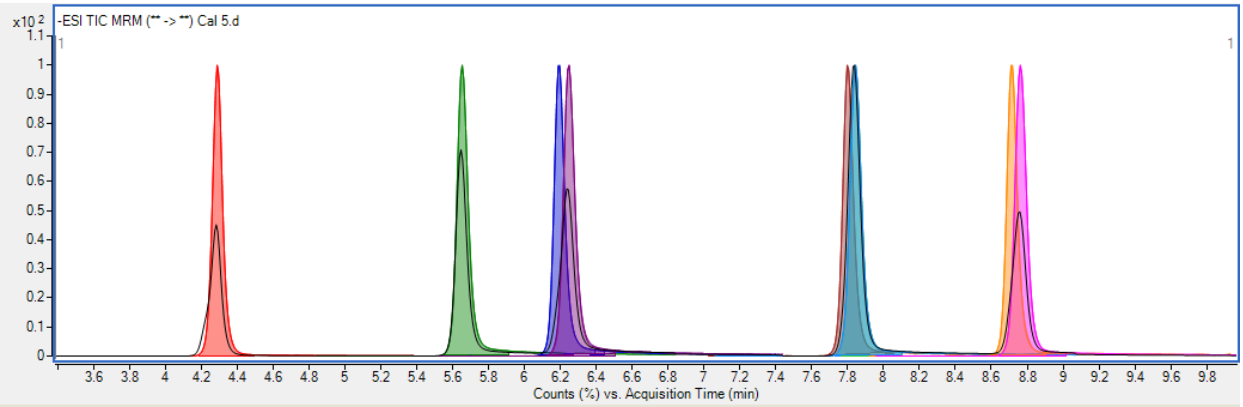
Table 2 Mass spectrometer parameters

Parameter	Setting
Ionization	Electrospray
Polarity	Negative
Gas Temperature	350°C
Drying Gas Flow	10.0 L/min
Nebulizer Pressure	40 psi
Capillary Voltage	4000 V

Table 3 DynamicMRM segment parameters

Compound	Precursor Ion	Product Ions	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)	RT (approx.)
Butabarbital	211.1	168	95	12	5	5.837
		42		15		
Butalbital	223.1	180.1	95	12	5	6.440
		42.1		16		
Butalbital-D_5	228.1	185.1	80	10	5	6.396
		42.1		12		
Pentobarbital/Amobarbital	225.1	182.2	95	12	5	8.065
		42.1		16		
Pentobarbital-D_5	230.2	187.1	85	18	5	8.022
		42.1		24		
Phenobarbital	231.1	188.2	95	4	5	4.386
		42.1		16		
Phenobatbital-D_5	236.1	193.1	95	0	5	4.445
		42.1		16		
Secobarbital	237.1	194.1	95	4	5	8.961
		42.1		12		
Secobarbital-D_5	242.2	199.2	100	0	5	8.927
		42.1		16		

The product ions in bold denote the quantifier transition. An example of the extracted ion chromatography from an extracted samples is shown in Figure 1.



Extraction Method

During method development, sample preparation procedures were evaluated. The first extraction procedure was the protein precipitation delineated in the Anti-Epileptic Drugs Quantitation and Confirmation by LCMSMS method in the Toxicology Procedure Manual. The extraction utilized 0.2 mL of biological fluid that was subsequently extracted with 1.0 mL of methanol. After precipitation, the samples were centrifuged and the topmost layer was transferred to autosampler vials for analysis. Upon investigation of this extraction, it was noted that the instrumental response for barbiturates was significantly low. Several instrumental

parameters were evaluated including injection volume and mobile phase composition. An increase in injection volume caused poor peak shape for phenobarbital.

To maintain the extraction and concentrate the samples to increase the instrumental response, the topmost layer after centrifugation was transferred to clean test tubes, dried down under nitrogen, and reconstituted. The reconstitution volume and solvent composition was evaluated. During the investigation, the dry down step required a significant amount of time and although reconstitution volume and composition were evaluated, the impact on the instrumental response was not significant enough to warrant the increased extraction time. Therefore, a liquid-liquid extraction was evaluated.

The liquid-liquid extraction evaluated utilized 0.1 mL of biological fluid and 400 µL of 1:9 n-hexane:ethyl acetate. The samples were vortexted for 5 minutes followed by centrifugation at approximately 2800 rpm for 15 minutes. The organic layer was transferred and dried down under nitrogen. The samples were then reconstituted in 100 µL of starting mobile phase. Preliminary experiments indicated promise with the extraction in comparison to the methanolic protein precipitation procedure. Slight modifications of the method included an evaluation into the vortexing time. Vortexing was initially performed for 5 minutes. Various vortexing times were evaluated and replicated to ensure that a decreased time maintained robustness and reproducibility. From this assessment, the extraction procedure was changed to an approximately 15 second vortex time. The shortest time point evaluated was 10 seconds which also produced comparable results to all other time segments tested.

Working Range

The working range was established to be 1 mg/L to 40 mg/L. The calibrators were prepared as delineated in Table 4.

Table 4 Instrumental method working range

Amount of 100 µg/mL stock solution (µL)	Amount of 10 µg/mL stock solution (µL)	Final concentration of Barbiturates (mg/L)
40		40
30		30
20		20
10		10
	50	5
	25	2.5
	10	1

The working range was evaluated using blank blood, antemortem blood, and postmortem blood. All three calibration curves produced consistent instrumental responses.

Accuracy (Bias) and Precision

During method development, accuracy and precision were evaluated. Three concentrations of fortified pooled blood samples were prepared in blank blood, antemortem blood, and postmortem blood. The samples were extracted in triplicate over two batch analyses. All quantitative results were based on extrapolation to a calibration curve prepared in blank blood. Accuracy and intermediate precision were calculated using Equation 1 and Equation 2.

Equation 1

$$Accuracy\ (\%) = \left|\left(\left(\frac{Mean\ of\ calculated\ values}{Expected\ value}\right) \times 100\%\right)\right|$$

Equation 2

$$Intermediate\ Precision\ (\%CV) = \left(\frac{Standard\ deviation\ of\ combined\ means}{Calculated\ grand\ mean}\right) \times 100\%$$

Accuracy and within-run precision were within ±20%. The accuracy for each compound and respective matrix is listed in Table 5.

Table 5 Barbiturate accuracy for blank blood, antemortem blood, and postmortem blood

Accuracy %Accuracy (SD); n=6									
	Blank Blood			Antemortem Blood			Postmortem Blood		
Compound	2.5 mg/L	10 mg/L	30 mg/L	2.5 mg/L	10 mg/L	30 mg/L	2.5 mg/L	10 mg/L	30 mg/L
Phenobarbital	94(1)	101(1)	97(2)	95(1)	100(1)	95(4)	96(1)	98(1)	94(3)
Butabarbital	91(2)	97(1)	96(2)	94(2)	97(1)	95(6)	95(1)	96(2)	93(4)
Butalbital	96(1)	100(1)	98(2)	96(1)	100(2)	96(7)	95(1)	98(2)	94(4)
Pentobarbital	96(2)	100(1)	97(3)	97(1)	100(2)	95(4)	96(1)	98(1)	94(3)
Secobarbital	97(2)	100(2)	97(3)	98(2)	99(2)	94(5)	96(2)	98(2)	93(3)

The accuracy for all compounds in all matrices evaluated was between 91±2% and 101±1% accuracy. In addition to pooled accuracy, intermediate precision was also evaluated in pooled blank blood, antemortem blood, and postmortem blood. The intermediate precision results are listed in Table 6.

Table 6 Barbiturate intermediate precision for blank blood, antemortem blood, and postmortem blood

	Intermediate Precision Average±SD(%CV); n=6								
	Blank Blood			Antemortem Blood			Postmortem Blood		
Compound	2.5 mg/L	10 mg/L	30 mg/L	2.5 mg/L	10 mg/L	30 mg/L	2.5 mg/L	10 mg/L	30 mg/L
Phenobarbital	2.36±0.04(2)	10.06±0.11(1)	29.16±0.51(2)	2.38±0.02(1)	10.02±0.12(1)	28.48±1.26(4)	2.39±0.02(1)	9.84±0.13(1)	28.23±0.77(3)
Butabarbital	2.27±0.04(2)	9.71±0.014(1)	28.81±0.71(2)	2.36±0.06(2)	9.72±0.13(1)	28.46±1.90(7)	2.37±0.02(1)	9.64±0.23(2)	28.03±1.06(4)
Butalbital	2.39±0.03(1)	10.03±0.12(1)	29.43±0.75(3)	2.41±0.03(1)	10.01±0.18(2)	28.86±1.98(7)	2.38±0.03(1)	9.83±0.19(2)	28.22±1.10(4)
Pentobarbital	2.41±0.06(2)	10.05±0.12(1)	29.247±0.78(3)	2.43±0.03(1)	9.98±0.17(2)	28.43±1.29(5)	2.41±0.02(1)	9.78±0.11(1)	28.29±0.99(4)
Secobarbital	2.42±0.04(2)	9.95±0.15(2)	29.242±0.76(3)	2.45±0.05(2)	9.94±0.17(2)	28.31±1.36(5)	2.40±0.05(2)	9.80±0.15(2)	27.97±0.86(3)

All compounds had a %CV equal to or less than 7%. The 30 mg/L pooled antemortem blood sample had the largest %CV for all targets. These samples demonstrated the most bias and imprecision of all pooled samples for all target compounds.

Ionization Suppression/Enhancement

Ionization suppression and enhancement was also evaluated during method development. A single source of blank blood, antemortem blood, and postmortem blood was evaluated for ionization suppression/enhancement. The evaluation was performed by assessing post-extraction fortified samples and neat standards in triplicate (per matrix source) at three concentrations (2.5 mg/L, 10 mg/L, and 30 mg/L). Ionization suppression/enhancement was calculated using Equation 3. Table 7 lists the average ionization suppression/enhancement for each compound within the analytical method.

Equation 3

$$Ion\ Suppression/Enhancement = \left(\frac{Average\ \ post-extraction\ fortified\ sample}{Average\ neat\ sample}\right) \times 100\%$$

Table 7 Ionization suppression/enhancement for blank blood, antemortem blood, and postmortem blood

	Ionization Suppression/Enhancement %Suppression/Enhancement(SD)		
Compound	Blank Blood	Antemortem Blood	Postmortem Blood
Phenobarbital	103(4)	102(5)	101(4)
Butabarbital	106(7)	102(6)	101(5)
Butalbital	101(5)	99(5)	101(4)
Pentobarbital	101(5)	99(5)	99(4)
Secobarbital	101(4)	99(6)	100(5)
Phenobarbital-D_5	102(2)	104(3)	99(4)
Butalbital-D_5	101(3)	102(5)	98(4)
Pentobarbital-D_5	101(2)	102(4)	98(3)
Secobarbital-D_5	101(3)	102(4)	98(4)

No significant ionization suppression or enhancement was noted for any compound within the three matrices evaluated. The percent ionization suppression/enhancement was between 98±4% to 106±7%. The amount of ionization suppression/enhancement was comparable between the target compounds and their associated internal standards.

Recovery

Recovery was evaluated by comparing the instrumental response of pre-extraction fortified and post-extraction fortified samples. The recovery of barbiturates in blank blood, antemortem blood, and postmortem blood was evaluated in triplicate (per matrix source) at three concentrations (2.5 mg/L, 10 mg/L, and 30 mg/L). Recovery was calculated using Equation 4. Table 8 lists the average recovery for each compound and internal standard in the three matrices evaluated.

Equation 4

$$Recovery = \left(\frac{Average\ pre - extraction\ spike}{Average\ post - extraction\ spike}\right) \times 100\%$$

Table 8 Recovery for blank blood, antemortem blood, and postmortem blood

	Recovery %Recovery(SD)		
Compound	Blank Blood	Antemortem Blood	Postmortem Blood
Phenobarbital	69(4)	69(5)	66(2)
Butabarbital	70(5)	70(6)	67(4)
Butalbital	70(5)	70(5)	66(3)
Pentobarbital	71(5)	70(5)	66(3)
Secobarbital	70(4)	69(5)	65(4)
Phenobarbital-D_5	68(4)	64(2)	64(3)
Butalbital-D_5	69(5)	65(1)	64(3)
Pentobarbital-D_5	70(4)	65(2)	64(4)
Secobarbital-D_5	70(4)	63(2)	63(5)

The recovery ranged from 63±5% to 71±5% for all compounds and internal standards. Secobarbital-D_5 in postmortem blood demonstrated the lowest recovery. The highest recovery was observed with pentobarbital in blank blood. The average recovery for target compound and associated internal standard was consistent.

Summary

A liquid-liquid extraction was developed for the quantitative analysis of barbiturates using LCMSMS in biological matrices. A negative ionization mode, DynamicMRM instrumental method was developed to evaluate a working range of 1 mg/L to 40 mg/L. Accuracy and precision, ionization suppression/enhancement, and recovery were evaluated for blank blood, antemortem blood, and postmortem blood during method development. Although all compounds were within

±20% accuracy, more specifically, all pooled samples were within 91±2% and 101±1% accuracy. No significant ionization suppression or enhancement was noted for the target compounds or internal standards within the method for any of the matrices evaluated. Additionally, the recovery was between 63±5% and 71±5% for all compounds in blank blood, antemortem blood, and postmortem blood. The conclusion of this method development suggests that the method is suitable to proceed on to full validation.

References

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